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Muscle contraction increases carnitine uptake via translocation of OCTN2

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**Running title:** Carnitine uptake during muscle contraction

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## ABSTRACT

Since carnitine plays an important role in fat oxidation, influx of carnitine could be crucial for muscle metabolism. OCTN2 (SLC22A5), a sodium-dependent solute carrier, is assumed to transport carnitine into skeletal muscle cells. Acute regulation of OCTN2 activity in rat hindlimb muscles was investigated in response to electrically induced contractile activity. The tissue uptake clearance ( $CL_{\text{uptake}}$ ) of L-[ $^3\text{H}$ ]carnitine during muscle contraction was examined *in vivo* using integration plot analysis. The  $CL_{\text{uptake}}$  of [ $^{14}\text{C}$ ]iodoantipyrine (IAP) was also determined as an index of tissue blood flow. To test the hypothesis that increased carnitine uptake involves the translocation of OCTN2, contraction-induced alteration in the subcellular localization of OCTN2 was examined. The  $CL_{\text{uptake}}$  of L-[ $^3\text{H}$ ]carnitine in the contracting muscles increased 1.4–1.7 fold as compared to that in the contralateral resting muscles ( $p < 0.05$ ). The  $CL_{\text{uptake}}$  of [ $^{14}\text{C}$ ]IAP was much higher than that of L-[ $^3\text{H}$ ]carnitine, but no association between the increase in carnitine uptake and blood flow was obtained. Co-immunostaining of OCTN2 and dystrophin (a muscle plasma membrane marker) showed an increase in OCTN2 signal in the plasma membrane after muscle contraction. Western blotting showed that the level of sarcolemmal OCTN2 was greater in contracting muscles than in resting muscles ( $p < 0.05$ ). The present study showed that muscle contraction facilitated carnitine uptake in skeletal muscles, possibly via the contraction-induced translocation of its specific transporter OCTN2 to the plasma membrane.

**Key words:** carnitine transporter, electrical stimulation, uptake clearance, subcellular localization, skeletal muscle

## Abbreviations

CL<sub>int</sub>, intrinsic clearance;

CL<sub>uptake</sub>, uptake clearance;

GAPDH, glyceraldehyde-3-phosphate dehydrogenase;

GasD, the deep portions of the gastrocnemius;

GasS, the surface portions of the gastrocnemius;

IAP, iodoantipyrine;

MCT4, monocarboxylate transporter 4;

OCTN2, carnitine/organic cation transporter;

OME, outer membrane-enriched fraction;

Pla, muscle plantaris;

Sol, muscle soleus

## INTRODUCTION

Carnitine is essential for the transport of long-chain fatty acyl groups into the mitochondrial matrix for subsequent beta-oxidation [1,2]. Another important role that carnitine plays in skeletal muscles is regulation of the acetyl coenzyme A/free coenzyme A ratio by buffering excess acetyl groups from pyruvate oxidation that occurs during events such as vigorous exercise [3,4]. However, acetylation depletes the free carnitine pool, and may impair free fatty acid transport into the mitochondria. Therefore, free carnitine availability is an important factor in skeletal muscle metabolism [4,5].

Carnitine metabolism during muscle contraction has not been well studied. Especially, its transport across the plasma membrane is not fully clarified. It is reported that during high-intensity exercise, there is a rapid depletion in the free carnitine level in the skeletal muscle, as free carnitine is used up in the production of acetylcarnitine [6]. Furthermore, previous studies in humans have revealed that carnitine metabolism and its exchange between the blood and muscle cells may occur during exercise. [5,7]. However, there is no evidence that muscle contraction per se augments carnitine uptake, and the regulatory mechanism of carnitine transport across the plasma membrane during muscle contraction remains unknown.

Carnitine uptake into cells is mediated by a sodium-dependent high-affinity transporter—organic cation transporter (OCTN2/SLC22A5) [8]. Since the carnitine concentration in the skeletal muscle (2–4 mmol kg<sup>-1</sup> wet muscle weight) is about 100 times higher than that in the plasma [9], carnitine should be transported into muscle cells against a considerable concentration gradient. Thus, the increase in plasma carnitine delivery per se (i.e., increased blood flow) does not account for an increase in carnitine influx into skeletal muscles. Recently,

we found that OCTN2 is expressed in rodent skeletal muscles, and comparison of various types of muscles indicated tight coupling of OCTN2 expression with carnitine uptake capacity of the muscle [10]. Therefore, OCTN2 could be responsible for the regulation of carnitine uptake during muscle contraction. However, our previous observation [10] was conducted on resting muscles, and carnitine uptake during muscle contraction, wherein tissue blood flow increases dramatically, still remains a question [11]. Therefore, in order to confirm that carnitine uptake by skeletal muscles during muscle contraction is regulated by OCTN2, and not by blood flow, we examined the effect of blood flow on tissue carnitine uptake.

It is well known that uptake of glucose and fatty acids increases when the transporters (GLUT4 and FAT/CD36, respectively) are translocated to the plasma membrane by stimulation of muscle contraction [12]. Whether a similar mechanism can promote carnitine uptake has been never demonstrated. On the basis of our immunohistochemical analysis, it is likely that OCTN2 can be translocated because OCTN2 localizes not only in the sarcolemma but also in the intracellular space (unpublished observation). Therefore, we investigated the hypothesis that muscle contraction induces upregulation of carnitine uptake, which is mediated by translocation of OCTN2 to the plasma membrane in this tissue. In the present study, muscle contraction was induced in a unilateral rat hindlimb by electrical stimulation, and the contralateral leg was considered as the resting leg. Integration plot analysis during muscle contraction was used to compare carnitine uptake clearance between the right and left legs. Furthermore, immunohistochemical analysis and were performed in order to detect changes in the subcellular localization of OCTN2 with/without muscle contraction. The present study is the first report to provide experimental evidence that contractile activity facilitates carnitine uptake through translocation of OCTN2 in the skeletal muscles.

## **MATERIALS AND METHODS**

### ***Animals***

Male Wistar rats weighing 249 – 337 g (n = 25) were used for the experiment. A standard diet (Oriental Yeast Co., Tokyo, Japan) and water were provided ad libitum. All procedures performed in this study were approved by the Ethics Committee on Animal Experimentation of Kanazawa University (protocol #: AP-101738).

### ***Materials***

L-[<sup>3</sup>H]Carnitine (84 Ci mmol<sup>-1</sup>) was purchased from GE Healthcare (Piscataway, NJ), and [<sup>14</sup>C]inulin (1–3 mCi g<sup>-1</sup>) was purchased from PerkinElmer Life Sciences (Boston, MA). [<sup>14</sup>C]iodoantipyrine (IAP) (55 mCi mmol<sup>-1</sup>) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO), Wako Pure Chemical Industries, Funakoshi Co. (Tokyo, Japan) and GE Healthcare (Piscataway, NJ).

### ***Muscle contraction protocol***

All surgical procedures were performed with the animals under pentobarbital sodium anesthesia (50 mg kg<sup>-1</sup> intraperitoneally). Under resting conditions, the left foot was attached to a clamp unit and connected to an electromotor system (Model RU-72, Nihon Koden, Tokyo, Japan). The right foot, with the contralateral resting muscle, was also clamped and lifted to the same height as the left foot. The ankle and knee joint were fixed at 90°. Isometric, tetanic muscle contractions were elicited in the left leg by electrostimulation via a surface electrode with trains

of stimuli (0.2-ms pulse duration at 40 Hz, 10 V, lasting for 0.2 s). The stimulation was applied at a rate of 1 contraction per second for 5 min.

### ***Experiment 1: Effect of muscle contraction on carnitine uptake***

#### ***Tissue uptake of carnitine***

The tissue distribution of L-[<sup>3</sup>H]carnitine was examined using the integration plot analysis as described previously [10,13]. Rats were anaesthetized, and isometric contraction of the left leg was induced by the method as described above section. Two minutes after the contraction started, L-[<sup>3</sup>H]carnitine (22.7 ng kg<sup>-1</sup>) with [<sup>14</sup>C]inulin (0.73 mg kg<sup>-1</sup>) dissolved in saline was injected via the left jugular vein. At designated times, blood was withdrawn from the right jugular vein, and plasma was separated by centrifugation. Three minutes later (5 min after stimulation started), immediately after the electrical stimulation stopped, the rats were sacrificed. The right and left hindlimb muscles (m. soleus (Sol), m. plantaris (Pla), and the surface and deep portions of m. gastrocnemius (GasS and GasD, respectively)) were excised immediately. Each tissue sample was weighed and solubilized with Soluene-350<sup>®</sup> (Packard Inc., Meriden, CT) at 55°C for 3 h. The solution was treated with hydrogen peroxide and neutralized with 5 M HCl. The solubilized tissues and plasma were each mixed with Clearsol I (Nacalai Tesque Inc., Kyoto, Japan) as a scintillation liquid, and the corresponding radioactivity was measured with a liquid scintillation counter, LSC-5100 (Aloka, Tokyo, Japan).

Tissue uptake clearance ( $CL_{\text{uptake}}$ ) of L-[<sup>3</sup>H]carnitine was measured by integration plot analysis as described previously [10]. Assuming that the efflux of the radioactive compounds from the tissue is negligible, the tissue uptake clearance ( $CL_{\text{uptake}}$ ) can be obtained using the following equation:



$$X_T(t)/C_p(t) = CL_{\text{uptake}} \cdot AUC_{(0-t)}/C_p(t) + V_0$$

where  $X_T(t)$  and  $C_p(t)$  are the amount and concentration of the compound in tissue and plasma, respectively, at time  $t$ .  $AUC_{(0-t)}$  is the area under plasma concentration curve from time 0 to  $t$ .  $V_0$  is the volume of distribution in which a rapid equilibrium with the plasma compartment.[10]

In order to examine the effect of blood flow on carnitine uptake in muscles, a separate group of rats was used to assess tissue blood flow by the  $CL_{\text{uptake}}$  of [ $^{14}\text{C}$ ]IAP. The blood flow rate was measured in the last 40 s of a 5-min muscle contraction by using the inert diffusible radiolabelled tracer [ $^{14}\text{C}$ ]IAP as described previously [14,15]. Briefly, the rats were anaesthetized and the muscle contraction procedure was performed for 5 min. Two minutes after the contraction started, L-[ $^3\text{H}$ ]carnitine (27.3 ng  $\text{kg}^{-1}$ ) was injected and blood was withdrawn from the right jugular vein at designated times. In the last 40 s of a 5-min muscle contraction, [ $^{14}\text{C}$ ]IAP (34.9 ng  $\text{kg}^{-1}$ ) dissolved in saline was intravenously injected. After intervals of 20 s and 35 s following injection, blood was withdrawn from the right jugular vein. After 40 s following injection, both the right and the left calf muscles were removed. The  $CL_{\text{uptake}}$  of [ $^{14}\text{C}$ ]IAP was obtained as the slope of  $X_T(t)/C_p(t)$  versus  $AUC/C_p(t)$  for [ $^{14}\text{C}$ ]IAP according to the above equation.

Furthermore, to evaluate carnitine uptake capacity of the muscles, intrinsic clearance ( $CL_{\text{int}}$ ) representing the influx of L-[ $^3\text{H}$ ]carnitine across the plasma membranes was calculated from the following equation using the well-stirred model [16]:

$$CL_{\text{int}} = \dot{Q} \cdot CL_{\text{uptake}} / (\dot{Q} - CL_{\text{uptake}})$$

where  $\dot{Q}$  represents the plasma flow rate, which was obtained as the  $CL_{\text{uptake}}$  of [ $^{14}\text{C}$ ]IAP.

## ***Experiment 2: Effect of muscle contraction on the sarcolemmal OCTN2 content***

### ***Isolation of outer membrane-enriched fraction***

Immediately after electrically induced muscle contraction, the right and left hindlimb muscles were excised. GasD samples were separated into 2 membrane fractions—outer membrane-enriched fraction (OME) and intracellular fraction—by a modified protocol based on the method reported by Juel et al. [19]. Briefly, the GasD was minced and incubated for 30 min in buffer A (2 M NaCl, 20 mM HEPES, and 0.2 mM PMSF; pH 7.4). After incubation, the tissue was recovered by centrifugation and homogenized in buffer B (250 mM sucrose, 1 mM EDTA, 20 mM HEPES, and 0.2 mM PMSF; pH 7.4) using a Polytron homogenizer (PT 1200 CL; Kinematica, Lucerne, Switzerland). The homogenate was centrifuged at 1,000 g for 5 min at 4°C, and then the supernatant was centrifuged at 10,000 g for 30 min at 4°C. The pellet (Fraction 1; F1) was resuspended in buffer B and saved. The supernatant was centrifuged again at 100,000 g for 30 min at 4°C. The pellet (Fraction 2; F2), the OME fraction, was resuspended in buffer B and used for western blotting. The supernatant (Fraction 3; F3) was also used. Isolation of sarcolemma was confirmed by Western blotting for monocarboxylate transporter 4 (MCT4), which is a plasma membrane protein.

### ***Western blotting***

Western blot analysis was performed as described previously [10]. Samples (10 µg lane<sup>-1</sup>) were separated by SDS-polyacrylamide gel electrophoresis on a 12% gel, and proteins were transferred to a polyvinylidene difluoride membrane (P-Membranes; ATTO, Tokyo, Japan). The membranes were blocked with Block Ace (DS Pharma Biomedical Co., Osaka, Japan) for 60 min at room temperature, and then incubated with appropriate primary antibodies. The polyclonal antibody to MCT4 was obtained from Chemicon (Temecula, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, MA) was used as a loading control.

### ***Experiment 3: Effect of muscle contraction on subcellular localization of OCTN2***

#### ***Hindlimb perfusion and fixation***

Under anesthesia, the hindlimb perfusion was performed as described previously [17]. In brief, the rat was killed by injection of 1 M KCl solution into the heart, and Krebs-Henseleit buffer including 100  $\mu$ M L-carnitine (Lonza Inc., VS, Switzerland) was perfused into the abdominal aorta in a flow-through mode at a constant flow (15 mL min<sup>-1</sup>). The flow rate was set at 22 mL min<sup>-1</sup>, and then isometric contraction was performed on the left hindlimb using the same protocol as described in the previous section. Immediately after muscle contraction, the hindquarters were perfused with 4% paraformaldehyde/phosphate buffer (pH 7.4) for 10 min.

#### ***Immunohistochemical analysis***

The fixed hindlimbs were gently removed and left in fresh fixation buffer at 4°C overnight. After dehydration with sucrose in phosphate-buffered saline (PBS), tissue blocks were frozen rapidly in isopentane cooled by liquid nitrogen. Frozen sections (6  $\mu$ m) of the isolated m. gastrocnemius were prepared as described previously [18]. Tissue sections were treated with Retrieve-All Antigen Unmasking System 1 (Covance, Princeton, NJ) for 10 min at 92°C to unmask the antigen. They were incubated with primary antibody diluted in blocking buffer at 4°C overnight and further incubated with secondary antibodies for 1 h at room temperature. The fluorescence was detected with a confocal laser scanning fluorescence microscope (LSM 710, Carl Zeiss, Göttingen, Germany).

#### ***Statistical analysis***

All data are expressed as mean  $\pm$  standard error of the mean (SEM). Paired *t*-test was used to compare the right and left leg muscles. Relationships between the selected variables were examined using the Pearson product-moment correlation coefficient. The level of significance was set at  $p < 0.05$ .

## RESULTS

### *Experiment 1: Effects of muscle contraction on carnitine uptake*

Figure 1 shows the  $CL_{\text{uptake}}$  of L-[ $^3\text{H}$ ]carnitine calculated from the integration plot in the 4 calf muscles of the legs under resting and contracting conditions. The data were corrected by the radioactivity of [ $^{14}\text{C}$ ]inulin, which cannot permeate the membrane easily and is a marker of vascular and interstitial fluids [10]. For Sol, GasS, and GasD, the  $CL_{\text{uptake}}$  of L-[ $^3\text{H}$ ]carnitine was significantly higher under contraction than under resting ( $p < 0.05$ ). For Pla, the  $CL_{\text{uptake}}$  tended to be greater under muscle contraction ( $p = 0.07$ ).

The  $CL_{\text{uptake}}$  of [ $^{14}\text{C}$ ]IAP and L-[ $^3\text{H}$ ]carnitine is shown in Table 1. These data were measured in separate animals from Figure 1, and the  $CL_{\text{uptake}}$  of L-[ $^3\text{H}$ ]carnitine were not corrected by [ $^{14}\text{C}$ ]inulin. In the resting condition, the  $CL_{\text{uptake}}$  of [ $^{14}\text{C}$ ]IAP was about 10-fold higher than that of L-[ $^3\text{H}$ ]carnitine. The  $CL_{\text{uptake}}$  of [ $^{14}\text{C}$ ]IAP was 5.0–8.0-fold higher in the contracting muscles than in resting muscles (Table 1). No association was observed between the changes in the  $CL_{\text{uptake}}$  of [ $^{14}\text{C}$ ]IAP and L-[ $^3\text{H}$ ]carnitine ( $r = 0.28$ ,  $p = 0.23$ ).

The  $CL_{\text{int}}$  of L-[ $^3\text{H}$ ]carnitine is also summarized in Table 1. For Sol, Pla, and GasD, the  $CL_{\text{int}}$  of L-[ $^3\text{H}$ ]carnitine was significantly higher by 1.17–1.32-fold under contraction than under resting ( $p < 0.05$ ). For GasS, however, the difference in the  $CL_{\text{int}}$  of L-[ $^3\text{H}$ ]carnitine was not statistically significant ( $p = 0.07$ ).

### *Experiment 2: Effects of muscle contraction on subcellular distribution of OCTN2*

Fractions of the muscle samples (GasD) obtained by the centrifugation procedure described above were characterized immunologically. Following exposure to a high-salt

concentration, it was possible to separate sarcolemmal and other membrane fraction [20]. In the present study, we confirmed that MCT4 was present only in the plasma membrane fraction (F2), and not in the other fractions (F1 and F3, Fig. 2A) when muscles were incubated in 2 M NaCl buffer. This indicated that optimal separation of membranes is achieved by exposing to high-salt buffer. We also confirmed that OCTN2 is present in both the plasma membrane and the intracellular fraction (Fig. 2B). Following 5 min of electrically induced muscle contraction, the OCTN2 content in the OME fraction (F2) was significantly increased (+49%,  $p < 0.05$ , Fig. 2B), suggesting that OCTN2 was translocated to the plasma membrane. As expected, muscle contraction had no effect on MCT4 redistribution (Fig. 2B).

### ***Experiment 3: Effects of muscle contraction on localization of OCTN2***

Immunohistochemical staining of OCTN2 demonstrated that the protein was located in or near the sarcolemmal membrane but was also present in the intracellular region (Figs. 3A). The control myofibers, which were not incubated with primary antibody, did not exhibit any labeling (data not shown).

In order to investigate changes in the localization of OCTN2 due to muscle contraction, cryosections taken from stimulated/unstimulated muscles (GasD) were immunofluorescently labeled. As shown in Fig. 3A and 3D, OCTN2 signals were higher on the sarcolemma and around the capillary vessels, especially in the contracting leg. Double staining with dystrophin as an outer membrane marker (Fig. 3B, 3E) further emphasized the colocalization of OCTN2 with dystrophin in contracting muscle (Fig. 3C, 3F).

## DISCUSSION

The present study is the first investigation to provide experimental evidence that contractile activity facilitates carnitine uptake through the translocation of OCTN2 in skeletal muscle. In agreement with our hypothesis, (1) electrically induced muscle contraction amplified carnitine uptake into muscle cells (Fig. 1, Table 1), but this uptake was independent of changes in blood flow; and (2) more OCTN2 protein localized to the plasma membrane in contracting muscle (Figs 2, 3). These results suggest that during muscle contraction, carnitine influx in the skeletal muscles is facilitated via contraction-induced changes in the subcellular localization of the carnitine transporter OCTN2.

In order to clarify the effect of muscle contraction on carnitine uptake, we performed electro-stimulation on rat hindlimb muscles and evaluated carnitine uptake clearance by *in vivo* integration plot analysis (Fig. 1). Our data clearly showed that carnitine uptake clearance was about 1.5 fold higher in contracting muscles than in resting muscles, suggesting that muscle contraction facilitated carnitine uptake into muscles. These data supports the hypothesis that exercise may activate transmembrane influx of carnitine as suggested by Vukovich et al. [7]. Some previous studies have also revealed an increase in the serum short-chain acylcarnitine concentration during exercise, indicating an efflux of acylcarnitine from the muscles to the blood [5,21]. These studies also showed that there is no change in the total muscle carnitine content, implying an exchange of free carnitine and carnitine esters between the muscle and blood during exercise [7]. The salutary effects of the facilitation of carnitine uptake and acetylcarnitine export during muscle contraction were associated with the regulation of acetylcarnitine/free carnitine ratio in sarcoplasm. Since muscle free carnitine availability becomes limiting to CPT1 during

exercise [6], the facilitation of carnitine turnover in contracting muscles is likely to be involved in the maintenance of intracellular free carnitine availability to sustain muscle fat oxidation. Although little attention has been paid to transmembrane carnitine transport during muscle contraction, the integration plot analysis performed in the present study proved that muscle contraction indeed increased carnitine uptake to maintain the free carnitine pool in skeletal muscle.

The blood flow did not affect the carnitine uptake in contracting muscle in the present study. In order to examine the impact of blood flow on muscle carnitine uptake, the [ $^{14}\text{C}$ ]IAP uptake clearance was evaluated. [ $^{14}\text{C}$ ]IAP is a rapid diffusible radiolabelled tracer that has been used as an index of blood flow [14,15]. The  $\text{CL}_{\text{uptake}}$  of [ $^{14}\text{C}$ ]IAP was considerably higher than that of L-[ $^3\text{H}$ ]carnitine even under the resting condition (8–10-fold difference, Table 1). Furthermore, relative change in both the uptake of [ $^{14}\text{C}$ ]IAP and L-[ $^3\text{H}$ ]carnitine did not correlate among the 4 calf muscles. We also evaluated the intrinsic clearance, which indicates the ability of the organ to take up the substrates from blood in the absence of other confounding factors (such as blood flow rate and protein binding). Our results demonstrated that the  $\text{CL}_{\text{int}}$  was 1.17–1.32-fold greater in the contracting muscles than in resting muscles (Table 1). Taken together, these observations proved that carnitine uptake during muscle contraction occurs independent of the change in blood flow, suggesting a specific regulatory mechanism via eg. carnitine transporter, even though our protocol only considers local blood flow regulation induced by electrical stimulation.

Immunoblot analysis revealed the change in OCTN2 concentration in the sarcolemma by a fractionation method [19]. We succeeded in extracting the OME fraction (confirmed by MCT4) and quantified the OCTN2 concentration. Our findings showed that muscle contractions



resulted in a 49% increase in the OCTN2 content of the sarcolemmal membrane as compared to control muscles (Fig. 2B). These biochemical data are consistent with the immunohistochemical observation that OCTN2 was intensified to the sarcolemma during muscle contraction (Fig. 3). Our hindlimb-perfused fixation technique allowed us to fix the intracellular proteins immediately after contraction. Transverse sections obtained from the fixed muscles were double stained with antibodies against both OCTN2 and dystrophin (a plasma membrane marker). Immunofluorescent staining indicated that OCTN2 was not localized exclusively in the sarcolemma of resting muscles. In contrast to the situation in the resting muscle, contracting muscles showed sarcolemma-specific expression of OCTN2 and an increased overlapping ratio of OCTN2 with dystrophin compared with basal muscles, suggesting translocation of OCTN2 to the sarcolemma due to contraction (Fig. 3). Taken together both biochemical and immunohistochemical observations supported the hypothesis that OCTN2 was translocated to the sarcolemma during muscle contraction.

In conclusion, this is the first study to show that muscle contraction facilitates carnitine uptake in skeletal muscles, independent of the changes in blood flow. Experimental evidence has shown that OCTN2 is redistributed from an intracellular location to the surface of the muscle, indicating that this protein is translocated in contracting skeletal muscle to upregulate carnitine uptake in skeletal muscle. The present study, therefore, provides new insight into the regulation of muscle energy metabolism in contracting skeletal muscles.

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## Figure Legends

Figure 1. Comparison between the muscle uptake clearance of L-[<sup>3</sup>H]carnitine by resting muscles (open column) and contraction-stimulated muscles (closed column). The data were corrected by the radioactivity of [<sup>14</sup>C]inulin, which is a marker of vascular and interstitial fluids. Sol: the soleus, Pla: the plantaris, GasS: surface portion of the gastrocnemius, GasD: deep portion of the gastrocnemius. Values are presented as means ± SEM (n = 5). \*: p < 0.05 vs. resting muscle.

Figure 2. Subcellular localization of OCTN2 and dystrophin in resting (A, B, C) and contraction-stimulated (D, E, F) muscles (deep portion of the gastrocnemius). In resting muscles, OCTN2 locates on the sarcolemma but also is detected in the intracellular space (A). In contrast to resting muscle, images of evoked-contraction muscles (D) show sarcolemma-specific expression of OCTN2. Dystrophin is the sarcolemmal marker (B and E). Compared with resting muscles (C), the overlap of OCTN2 (red) and dystrophin (green) is increased in contracting muscles (F) (indicated by arrowheads). Scale bar = 50 μm.

Figure 3. Comparison of OCTN2 expression in the intracellular fraction and the OME fraction with/without muscle contraction.

A: Representative western blot of the plasma membrane marker (MCT4) in Fractions 1–3. When skeletal muscles (GasD) were incubated in high-salt buffer [indicated with a plus (+) sign], MCT4 was detected in Fraction 2 but not in

Fractions 1 or 3.

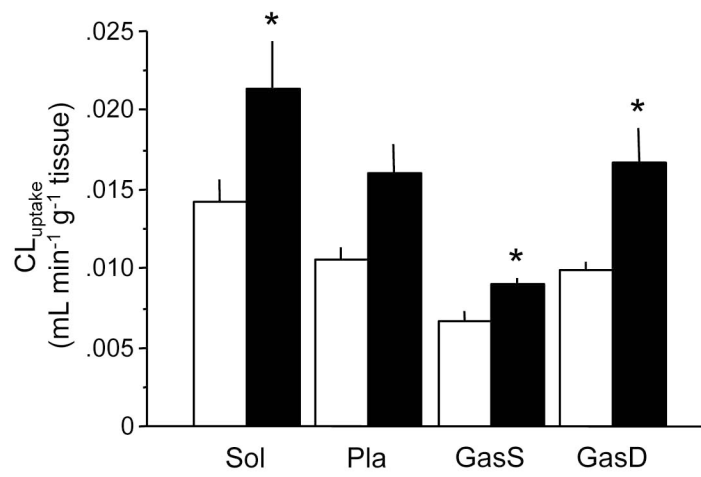
B: Effect of muscle contraction on OCTN2 and MCT4 protein expression in the intracellular fraction and the OME fraction obtained from resting muscles (open column) and from contracting muscles (closed column). Representative immunoblots are shown above the quantified data. The amount of OCTN2 is normalized to GAPDH level. Quantification of OCTN2 for the contracting muscles is expressed relative to the contralateral resting muscles from the same animals. OME, outer membrane-enriched fraction. Values are presented as means  $\pm$  SEM (n = 6). \*:  $p < 0.05$  vs. resting muscle.

Table 1. Tissue uptake clearance of L-[ $^3\text{H}$ ]carnitine and [ $^{14}\text{C}$ ]IAP and Intrinsic clearance.  
Unit:  $\text{mL min}^{-1} \text{g}^{-1}$  tissue (mean  $\pm$  SEM), n = 5, Sol: the soleus, Pla: the plantaris, GasS: surface portion of the gastrocnemius, GasD: deep portion of the gastrocnemius, IAP: iodoantipyrine. \*:  $p < 0.05$  vs. resting muscle.

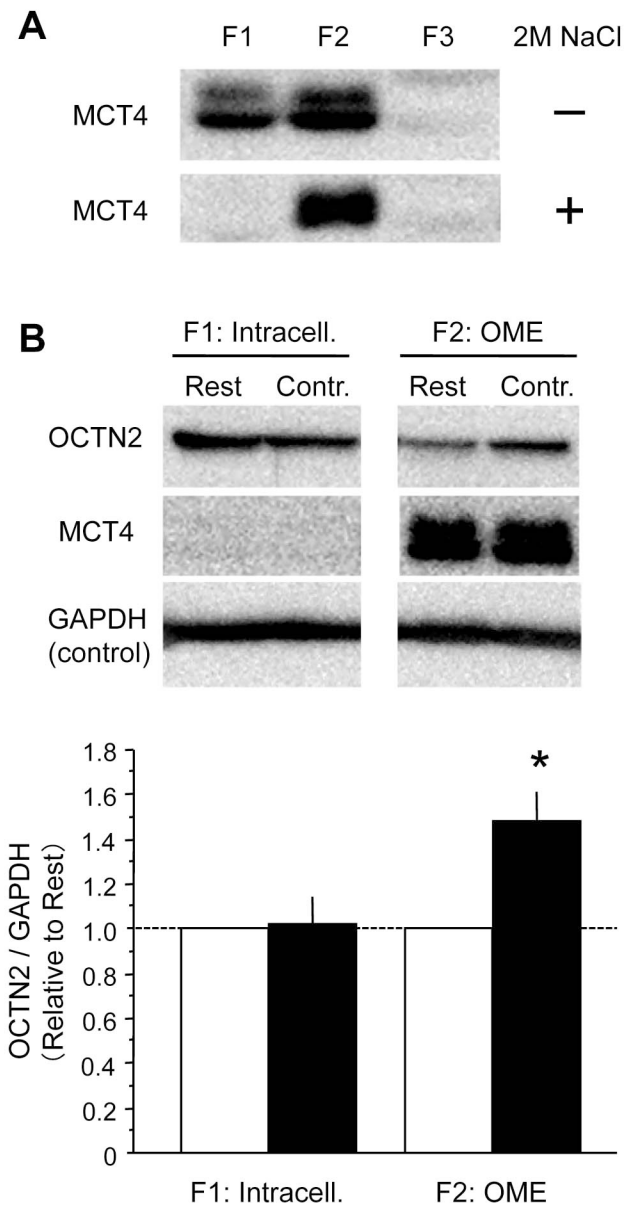
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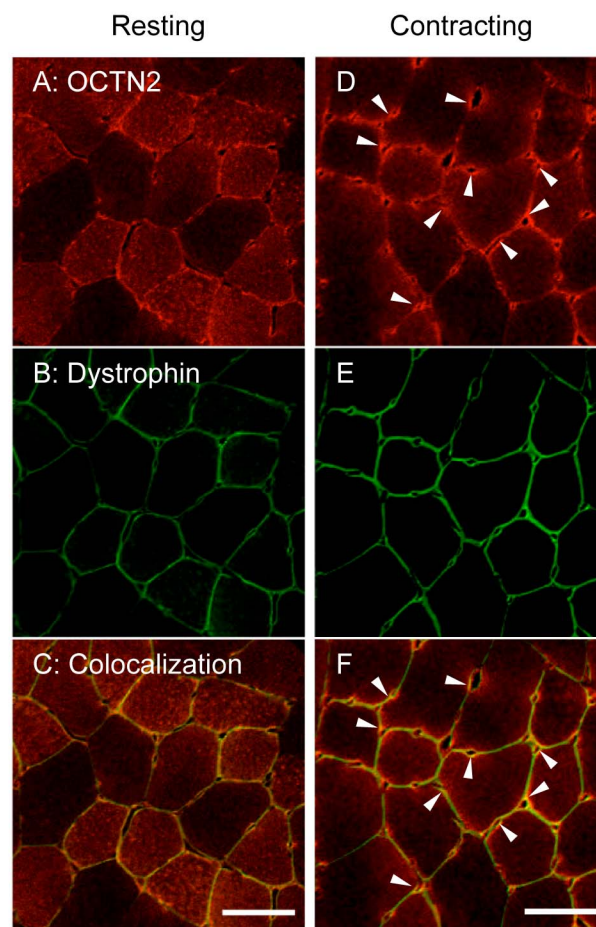


**Figure 1.**  
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**Figure 2.**  
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**Figure 3.**  
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Table 1. Tissue uptake clearance of L-[<sup>3</sup>H]carnitine and [<sup>14</sup>C]IAP and Intrinsic clearance.

Muscle	CL <sub>uptake</sub> of L-[ <sup>3</sup> H]carnitine		CL <sub>uptake</sub> of [ <sup>14</sup> C]IAP		CL <sub>int</sub> of L-[ <sup>3</sup> H]carnitine	
	Rest	Contraction	Rest	Contraction	Rest	Contraction
Sol	0.030 ± 0.001	0.044 ± 0.002*	0.305 ± 0.091	1.223 ± 0.071*	0.034 ± 0.001	0.045 ± 0.002*
Pla	0.023 ± 0.000	0.031 ± 0.001*	0.222 ± 0.029	1.221 ± 0.088*	0.026 ± 0.000	0.031 ± 0.001*
GasS	0.017 ± 0.000	0.023 ± 0.001*	0.136 ± 0.015	1.068 ± 0.078*	0.020 ± 0.000	0.023 ± 0.001
GasD	0.026 ± 0.001	0.037 ± 0.002*	0.255 ± 0.052	1.186 ± 0.190*	0.030 ± 0.001	0.038 ± 0.002*

Unit: mL min<sup>-1</sup> g<sup>-1</sup> tissue (mean ± SEM), n = 5, Sol: the soleus, Pla: the plantaris, GasS: surface portion of the gastrocnemius, GasD: deep portion of the gastrocnemius, IAP: iodoantipyrine. \*: p < 0.05 vs. resting muscle.

## Table 1.

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